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Analysis of the HIV-1 LTR NF-κB-Proximal Sp Site III: Evidence for Cell Type-Specific Gene Regulation and Viral Replication

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It has been widely demonstrated that the human immunodeficiency virus type 1 (HIV-1) envelope, specifically the V3 loop of the gp120 spike, evolves to facilitate adaptation to different cellular populations within an infected host. Less energy has been directed at determining whether the viral promoter, designated the long terminal repeat (LTR), also exhibits this adaptive quality. Because of the unique nature of the cell populations infected during the course of HIV-1 infection, one might expect the opportunity for such adaptation to exist. This would permit select viral species to take advantage of the different array of conditions and factors influencing transcription within a given cell type. To investigate this hypothesis, the function of natural variants of the NF-κB-proximal Sp element (Sp site III) was examined in human cell line models of the two major cell types infected during the natural course of HIV-1 infection, T cells and monocytes. Utilizing the HIV-1 LAI molecular clone, which naturally contains a high-affinity Sp site III, substitution of low-affinity Sp sites in place of the natural site III element markedly decreased viral replication in Jurkat T cells. However, these substitutions had relatively small effects on viral replication in U-937 monocytic cells. Transient transfections of HIV-1 LAI-based LTR-luciferase constructs into these cell lines suggest that the large reduction in viral replication in Jurkat T cells, caused by low-affinity Sp site III variants, may result from reduced basal as well as Vpr- and Tat-activated LTR activities in Jurkat T cells compared to those in U-937 monocytic cells. When the function of Sp site III was examined in the context of HIV-1 YU-2-based LTR-luciferase constructs, substitution of a high-affinity element in place of the natural low-affinity element resulted in increased basal YU-2 LTR activity in Jurkat T cells and reduced activity in U-937 monocytic cells. These observations suggest that recruitment of Sp family members to Sp site III is of greater importance to the function of the viral promoter in the Jurkat T cell line as compared to the U-937 monocytic cell line. These observations also suggest that other regions of the LTR may compensate for Sp recruitment defects in specific cell populations.

INTRODUCTION

Isolates of human immunodeficiency virus type 1 (HIV-1) exhibit marked differences in their ability to replicate in primary macrophages and T cell lines *in vitro.* Macrophage-tropic (M-tropic) non-syncytium-inducing (NSI) variants of HIV-1 replicate efficiently in primary T cells and macrophages but not in transformed T cell lines. T-cell-tropic (T-tropic) syncytium-inducing (SI) variants of HIV-1 replicate efficiently in primary T cells and in transformed T cell lines but not in primary macrophages. Restricted replication in these cell types is frequently associated with viral entry, a process regulated by interactions between the viral envelope gene product (gp 120) and CD4 in conjunction with certain chemokine receptors (reviewed in Berger *et al.,* 1999 and Clapham, 1999). Sequence alterations in the V3 loop of gp120 are thought to facilitate changes in cellular tropism by altering the affinity for particular coreceptor molecules (O'Brien *et al.,* 1990; Hwang *et al.,* 1991; Shioda *et al.,* 1991). HIV-1 variants containing V3 loop sequences recognizing the CCR5 chemokine coreceptor (designated R5 strains) are strongly correlated with the M-tropic, non-syncytium-inducing phenotype, whereas variants containing sequences recognizing the CXCR4 chemokine coreceptor (designated X4 strains) are strongly correlated with the T-tropic, syncytium-inducing phenotype (Speck *et al.,* 1997).

Although viral entry is critical for replication to proceed, there are indications that HIV-1 cellular tropism is not solely determined by the structure of the viral gp120. Chimeric viral constructs containing the V3 region of an M-tropic R5-dependent envelope (strain 89.6) in a T-tropic X4-dependent viral backbone (strain HXB) indicated that, although entry into primary macrophages was mediated by the R5 V3 loop, replication was blocked at a postentry step (Smyth *et al.,* 1998). Replication approaching that of the parental 89.6 construct was achieved in an HXB-based chimeric construct upon transfer of a 2.7-kb region of 89.6 genome containing *tat, rev, vpu, env, nef,* and the 3′ U3 region of the LTR (Kim *et al.,* 1995). Although subparental levels of replication were achieved

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following the exchange of smaller regions of the 89.6 genome, complete elimination of the postentry block, as shown by Smyth and coworkers (1998), required the transfer of multiple nonenvelope factors, suggesting that several nonenvelope factors may play an important role in generating the M-tropic phenotype. In addition, a more recent report has shown that X4 variant viruses can enter macrophages utilizing low levels of naturally expressed CXCR4 chemokine receptor, but fail to initiate a productive replication cycle as the result of a postentry block (Schmidtmayerova *et al.,* 1998). The results indicated that two non-envelope-based factors contributed to the block. Specifically, the X4 variant viruses exhibited an alteration in the kinetics of reverse transcription and a fivefold reduction in nuclear import as compared to R5 viruses.

HIV-1 cellular tropism may play an important role in disease progression. The primary and clinically latent stages of infection are associated with viral species exhibiting M-tropic NSI replication characteristics (Schuitemaker *et al.,* 1991; Valentin *et al.,* 1994), whereas clinical disease progression is associated with the development of viral species exhibiting T-tropic SI replication characteristics (Connor and Ho, 1994; Schuitemaker *et al.,* 1992). The *in vivo* adaptation of M-tropic viral species to a species exhibiting more T-tropic characteristics is likely driven, in part, by the selective pressure of the host immune system acting in conjunction with the natural variation of the HIV-1 genome (Delwart *et al.,* 1994; Lukashov *et al.,* 1995). This process is facilitated by the ability of the virus to generate genomic sequence variation through a high level of viral replication coupled with the low fidelity of the viral reverse transcriptase enzyme (Pang *et al.,* 1992; Ewald, 1994).

A potential site for postentry adaptation is the viral long terminal repeat (LTR), which, in its 5′ position in the proviral genome, serves as the promoter for viral gene expression (Cullen, 1991; Jones and Peterlin, 1994). Interactions between the *cis*-acting elements present in the viral promoter and cellular transcription factors present in the host cell influence the rate of viral gene expression under a wide variety of conditions including cellular stimulation, division, and differentiation. The core viral promoter is comprised of a TATAA box and three Sp family binding sites. This region is necessary for efficient basal transcription and thus essential for the function of HIV-1 proviral DNA-mediated gene expression. Immediately upstream of the three Sp elements is the enhancer region, comprising two inducible NF-κB binding sites. This region, although not absolutely necessary for viral replication, responds to cellular activation signals by stimulating LTR activity and increasing the rate of viral production. Upstream of the enhancer region lies the modulatory region, which contains numerous transcription factor binding sites that may increase or decrease transcription. LTR activity is also enhanced by the viral Tat protein (Arya *et al.,* 1985). Tat-mediated transactivation of the LTR requires an interaction between Tat and an RNA stem-loop structure, termed the transactivation response element (TAR) (reviewed by Jones and Peterlin, 1994). Tat transactivation may also be influenced by Sp elements in the core promoter. The loss of Sp elements in the HIV-1 LTR dramatically reduces Tat-mediated LTR activity (Harrich *et al.,* 1989). In addition, the Tat-inducibility of certain synthetic promoters has been shown to be Sp1-dependent (Kamine *et al.,* 1991). Sp sites within the viral core promoter have also been shown to be important in mediating LTR transactivation by the viral accessory protein Vpr (Wang *et al.,* 1995). Overall, Sp elements within the core viral promoter have been shown to comprise a critical region of the viral promoter and thus represent at least one region, which could be subject to sequence variation that facilitates viral adaptation to a given cellular environment, leading to enhanced viral replication under select intracellular conditions.

Several studies have reported that the LTR may evolve independently in different tissues, thereby implicating the LTR as a potential contributor to cell type-specific gene expression and viral replication. Some evidence, though indirect, was provided by phylogenetic analyses of HIV-1 sequences derived from different tissues (Ait-Khaled *et al.,* 1995; Corboy and Garl, 1997), as well as a functional approach that examined tissue- and cell type-specific LTR-directed transgene expression in mice (Corboy *et al.,* 1992). More direct evidence of LTR involvement in cell type-specific viral replication was provided by the finding that viral replication in primary monocytes, but not primary T cells, requires the presence of intact C/EBP sites within the viral LTR (Henderson and Calame, 1997). These findings demonstrated that *cis*-acting elements within the LTR may exhibit different patterns of utilization in T cells and monocytes, evidence that is consistent with the hypothesis that cell type-specific transcription factor or factor combinations can be utilized by properly adapted viral promoter structures to contribute to HIV-1 tropism.

Evidence presented herein suggests that the relative affinity of the NF-κB-proximal Sp element for Sp factors and its impact on viral replication is influenced by the cell type in which the virus replicates. These studies indicate that weak Sp binding sites are of greater detriment to virus replication in the Jurkat T cell line than they are to virus replication in the U-937 monocytic cell line. Additional evidence is presented which suggests that the impact on viral replication can be attributed to cell type-specific differences in basal, as well as Vpr- and Tat-activated, LTR activity.

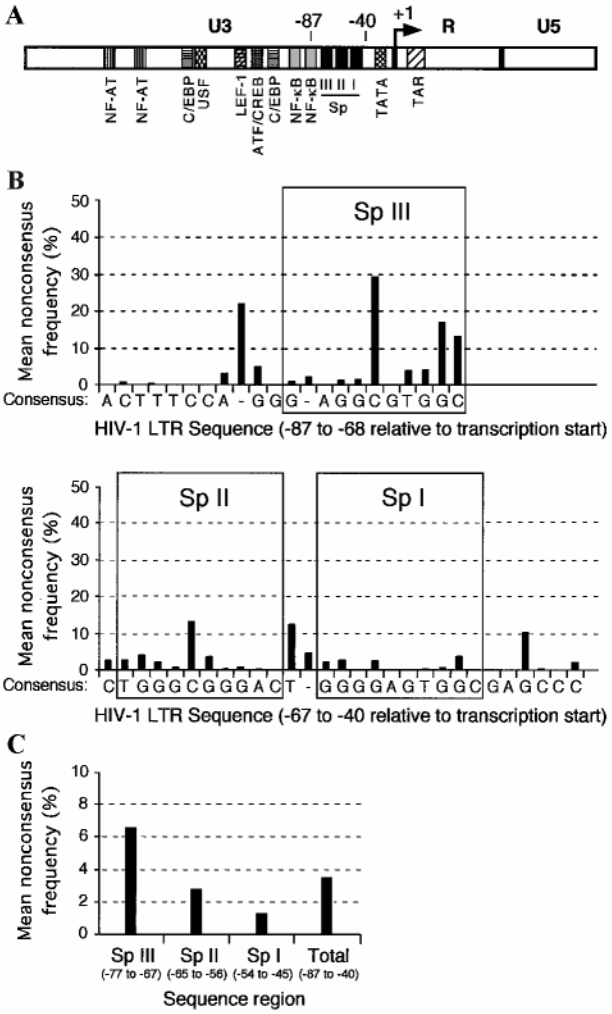


FIG. 1. Location of *cis*-acting Sp elements in the HIV-1 LTR and relative conservation of nucleotide sequence. (A) Relative location of the Sp sites and additional elements in the HIV-1 LTR. Not all the elements described in the literature are indicated. (B) LTR sequences were aligned and compared to the clade B consensus sequence. The frequency of occurrence of a non-clade B nucleotide was calculated at each nucleotide position for each individual as a percentage of total LTRs examined from each individual. The mean nonconsensus frequency (NCF) was calculated from these values at each nucleotide position for the population of individuals and plotted above the consensus sequence. The nucleotide changes most often observed were apparent nucleotide substitutions, although insertions and deletions did occur at certain positions and these were also included as a nucleotide change in this analysis. (C) The mean nonconsensus frequencies for each 10-bp Sp site and the entire Sp region are shown.

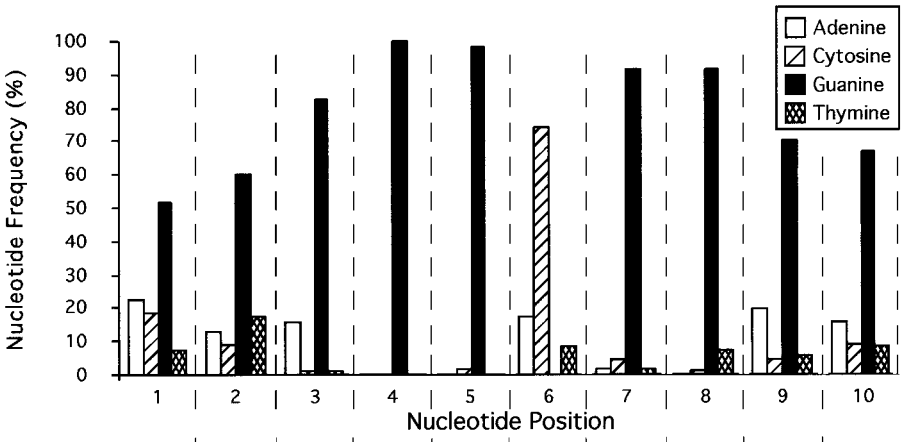
RESULTS

The G/C-rich elements of PBMC-derived HIV-1 LTRs exhibit a high degree of nucleotide sequence conservation

The location of the Sp array in the HIV-1 LTR is shown relative to additional transcriptional control elements (Fig. 1A). To investigate the general level of nucleotide variation within the G/C-rich region containing the *cis*-acting Sp elements, PBMC-derived LTR sequences from three large published studies were compiled and analyzed (Estable *et al.,* 1996; Kirchhoff *et al.,* 1997; Michael *et al.,* 1994). Each study contained multiple LTRs derived from four or more individuals that resided within North America for a total of 150 sequences derived from 45 individuals. The analysis was conducted by first examining the LTRs of each individual separately, to calculate the frequency of nonconsensus nucleotides at each nucleotide position as a percentage of total LTRs examined from the individual. The mean frequency of nonconsensus nucleotides of all individuals (nonconsensus frequency, NCF) at each nucleotide position in the Sp array region was determined. This information was plotted as a bar graph above the clade B consensus sequence (Fig. 1B).

At each nucleotide position in the array (including flanking sequence at both ends), the most common nucleotide matched the consensus for HIV-1 clade B, the most common HIV-1 subtype in North America. Per nucleotide NCFs were averaged over the entire Sp array (Sp site III, II, and I, including flanking sequence) and within individual 10-bp Sp sites, to express an overall level of sequence variation for both the region and each individual site (Fig. 1C). Over the entire region, variation within the Sp array was shown to be very low, with a mean regional NCF of 3.4% (Fig. 1C). By way of comparison, the 3′ NF-κB element exhibited a mean NCF of 6.3% (data not shown). The highly conserved nature of this region underscores its importance in viral gene regulation and replication. Of the three individual sites, variation is lowest in site I (1.3% mean NCF), intermediate in site II (2.7% mean NCF), and highest in site III (6.6% mean NCF). Since it was of interest to determine how natural sequence variation might affect viral gene expression and replication in the different cell populations which typically serve as hosts for HIV-1 infection, the impact of sequence variation within the most variable element, the NF-κB-proximal Sp site III, was selected for initial studies.

To identify naturally occurring HIV-1 LTR Sp site III variants which were likely to impact factor recruitment and transcriptional function, the Sp consensus binding site derived from analysis of 108 published Sp factor binding sites (TRANSFAC) (Heinemeyer *et al.,* 1999) was examined (Fig. 2). The strongest conservation occurs near the center of the Sp binding site at positions 3–6 (GCGG) within the 10-bp element. With the exception of the central C nucleotide (position 5), which is often a T or an A but never a G, the surrounding three guanine nucleotides are conserved in nearly all known Sp binding sites. Moreover, the guanine residue at position four of the consensus Sp site appeared to be imperative for maximizing factor recruitment, since no studies of effective Sp binding sites containing any other nucleotide at this position have been reported. Based on this informa-



Sp Consensus	G	G	G	G	G	C	G	G	G	G
Clade B Cons.	G	G	A	G	G	C	G	T	G	G
LIII	G	G	A	G	G	C	G	T	G	G
YIII	G	G	A	A	G	C	G	T	G	G
p4del	G	G	G	A	G	C	G	T	G	G

FIG. 2. Comparison of consensus Sp binding element with respect to the HIV-1 Sp elements examined. Bar graph depicts the frequency of each nucleotide encountered at a given position within the consensus Sp binding element. Also depicted are four HIV-1 elements: the clade B consensus Sp site III (Clade B cons.), the LAI-derived Sp site III (LIII), the YU-2-derived Sp site III (YIII), and a natural variant Sp site III containing sequences similar to those found in YU-2 (p4del). By comparing the positions of nucleotide alterations within the Sp site III variants to the frequencies of such alterations within the consensus Sp element one can estimate the impact of a given nucleotide alteration on factor recruitment.

tion, two variant sites that exhibit alterations of the guanine at position four were selected. Specifically, the Sp site III from HIV-1 YU-2, a brain-derived M-tropic R5-dependent molecular clone (Li *et al.,* 1991), and the p4del natural variant, a sequence variant found in LTRs amplified from lung tissue of an HIV-1-infected patient (Ait-Khaled *et al.,* 1995), were used in subsequent studies (Fig. 2).

Selected Sp site III variants exhibit weak Sp factor recruitment

The Sp family of transcription factors includes a number of closely related proteins, including Sp1, Sp3, and Sp4. To identify the specific Sp family members present in U-937 monocytic and Jurkat lymphocytic nuclear extract, electophoretic mobility shift (EMS) analyses were conducted with the high-affinity clade B consensus Sp site III (LIII, Fig. 3A) as a radiolabeled probe. In both cases, four distinct DNA–protein complexes were observed (Fig. 3B; lanes 1 and 6). In the presence of antibody directed against Sp1, a majority of complex A was replaced by a lower-mobility complex (Fig. 3B; lanes 2 and 7). Complexes B and C were shown to be reactive with antibody directed against Sp3 by abrogation or abrogation coupled with mobility shift (Fig. 3B; lanes 3 and 8). Consistent with the analysis of individual antibodies, the use of both Sp1 and Sp3 antisera resulted in the shift or abrogation of complexes A, B, and C (Fig. 3A; lanes 4 and 9). Sp4 antisera did not react significantly with any of the observed complexes (Fig. 3A; lanes 5 and 10). Complex D was unaffected by the presence of any of the Sp antisera examined, and has been shown to be nonspecific by competition with homologous and nonhomologous competitors (data not shown).

The ability of selected naturally occurring variants of HIV-1 Sp site III to bind Sp family transcription factors was examined *in vitro* utilizing both direct binding and competition EMS analysis. First, U-937 monocytic and Jurkat lymphocytic nuclear extracts were reacted with radiolabeled probes containing the clade B consensus Sp site III, the YU-2-derived Sp site III, and the p4del variant Sp site III (Fig. 3A; LIII, YIII, and p4del, respectively). Strong Sp1- and Sp3-containing DNA–protein complexes were observed when the LIII probe was reacted with either U-937 or Jurkat nuclear extracts (Fig. 3C; lanes 1 and 4). In the presence of either nuclear extract, the use of the variant Sp site III elements (YIII and p4del) resulted in a significant reduction in complex formation as compared to the clade B consensus (LIII) Sp element (Fig. 3C; compare lane 1 to lanes 2 and 3, and lane 4 to lanes 5 and 6). Although the use of variant Sp site III elements (YIII and p4del) resulted in the generation of little or no Sp-containing DNA–protein complexes in the presence of U-937 nuclear extract (Fig. 3C; lanes 2 and 3), low-intensity Sp-containing DNA–protein complexes were observed in the presence of Jurkat nuclear extract (Fig. 3C; lanes 5 and 6). In addition, Sp-related complex formation was greater in the presence of the p4del Sp site III element than in that of the YIII element. This difference was readily apparent in

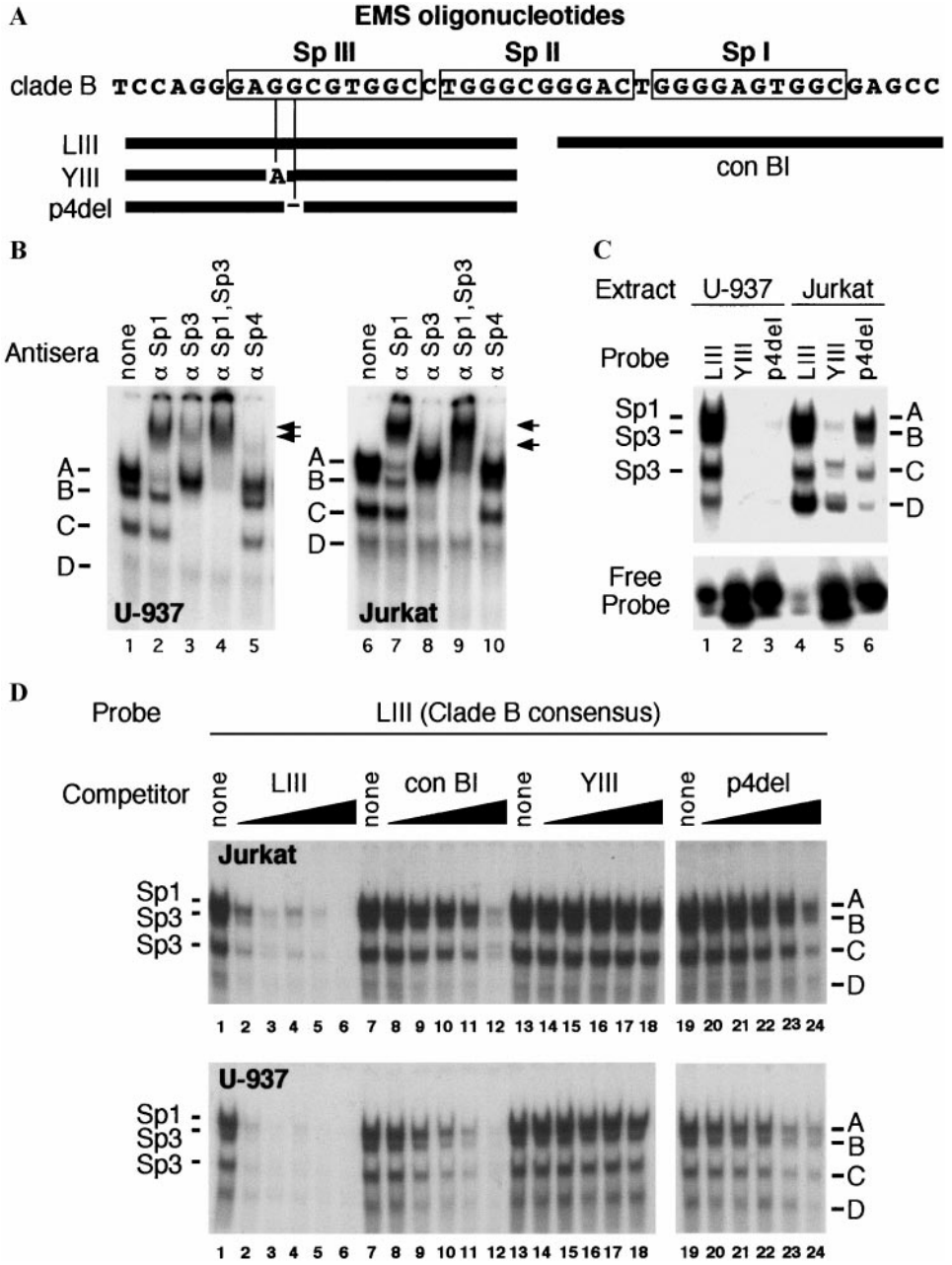


FIG. 3. Comparison of the affinity of Sp factors for different Sp site variants by competition EMS analyses. (A) The sequences of the four DNA oligonucleotides used in EMS analyses are represented by bars drawn below the clade B consensus sequence. Changes from the consensus are indicated with the nucleotide substitution shown. A dash indicates a deletion of the nucleotide. EMS analyses using the HIV-1 clade B consensus Sp site III were performed as described under Materials and Methods. (B and C) Binding reactions included 6 μg of U-937 or Jurkat nuclear extract with 1 ng radiolabeled probe (approximately 60,000 cpm). Antisera (1 μl) was added as indicated. DNA–protein complexes are labeled A through D, whereas supershifted DNA–protein complexes are indicated with arrows. (D) An equal amount of nuclear extract (6 μg) derived from Jurkat or U-937 cells was incubated with labeled clade B consensus Sp site III probe (0.5 ng) in all reactions. Specific competitor oligonucleotides were added to reactions as indicated at molar equivalents of 5-, 25-, 50-, 100-, and 200-fold excess compared to labeled probe in each reaction. The competitors correspond to the LAI-derived Sp site III, which is equivalent to the clade B consensus Sp site III (LIII, lanes 2–6), the clade B consensus Sp site I (con BI, lanes 8–12), the YU-2-derived Sp site III (YIII, lanes 14–18), and the p4del Sp site III (p4del, lanes 19–24).

reactions containing Jurkat nuclear extract (Fig. 3C; compare lanes 5 and 6), and could also be detected in reactions containing U-937 nuclear extract following longer autoradiographic exposures (data not shown). Hence, the p4del Sp site III appears to be intermediate between the clade B consensus and the YIII element in its ability to recruit Sp factors. In addition to quantitative differences in Sp-related complex formation, the Sp3-containing complex C exhibited alterations in mobility, which were dependent on the Sp site III probe utilized

(Fig. 3C; compare lanes 5 and 6). The observed difference in DNA–protein complex formation between the two variant Sp elements may be the result of a sequence-dependent binding of the two recently proposed Sp3 isoforms (Suske, 1999; Kennett *et al.,* 1997)

To more clearly examine the relative ability of each Sp site III element to bind Sp factors, the high-affinity clade B consensus Sp site III (LIII), the low-affinity clade B consensus site I (con BI), the YU-2-derived Sp site III (YIII), and the p4del variant Sp site III (p4del) were used as competitor DNAs with respect to factor binding to the radiolabeled clade B consensus Sp site III element (LIII). These studies were performed using nuclear extracts derived from Jurkat T cells and U-937 monocytic cells. In both cases, the clade B consensus Sp site III acted as a highly efficient competitor, while the low-affinity clade B consensus site I acted as a very inefficient competitor (Fig. 3B; compare LIII, lanes 1–6, to con BI, lanes 7–12). Consistent with the direct binding studies, the YU-2-derived Sp site III exhibited little, if any, ability to compete with the clade B consensus probe using either Jurkat or U-937 nuclear extracts (Fig. 3B; YIII, lanes 13–18). The p4del variant Sp site III competed less efficiently than the low-affinity clade B consensus site I, but more efficiently than the YU-2-derived Sp site III (Fig. 3B; compare p4del, lanes 19–24, to con BI and YIII, lanes 7–12 and 13–18, respectively). In summary, both the YU-2-derived and p4del variant Sp site III exhibited binding phenotypes characterized by weak factor recruitment.

Replication of recombinant LAI-based HIV-1 molecular clones containing the YU-2 and p4del Sp site III configurations

To directly determine whether the YU-2 and p4del Sp sites alter viral replication, these elements were introduced into the HIV-1 LAI molecular clone and viral replication was examined in the Jurkat lymphocytic and U-937 monocytic cell lines. In Jurkat cells, the replication rate of both mutant viruses was significantly lower than that of the parental LAI strain (Fig. 4A). The LAI-p4 HIV-1 variant exhibited a 6-day lag phase before detectable levels of p24 were produced. Although this delay does not appear to significantly alter the slope of the viral replication curve, it was associated with a reduction in the magnitude of p24 production by nearly 3 logs between mutant and parental viruses at each time point examined. The LAI-YIII virus did not generate any detectable p24 production at the time points examined. These data clearly demonstrate that the rate of HIV-1 replication in the T cell line was severely affected when the high-affinity clade B consensus Sp site III was converted to an element with lower affinity. However, when this experiment was performed in the U-937 monocytic cell line, the replication of the parental and mutant viruses was more comparable (Fig. 4B). At Day 12 postinfection the LAI-p4

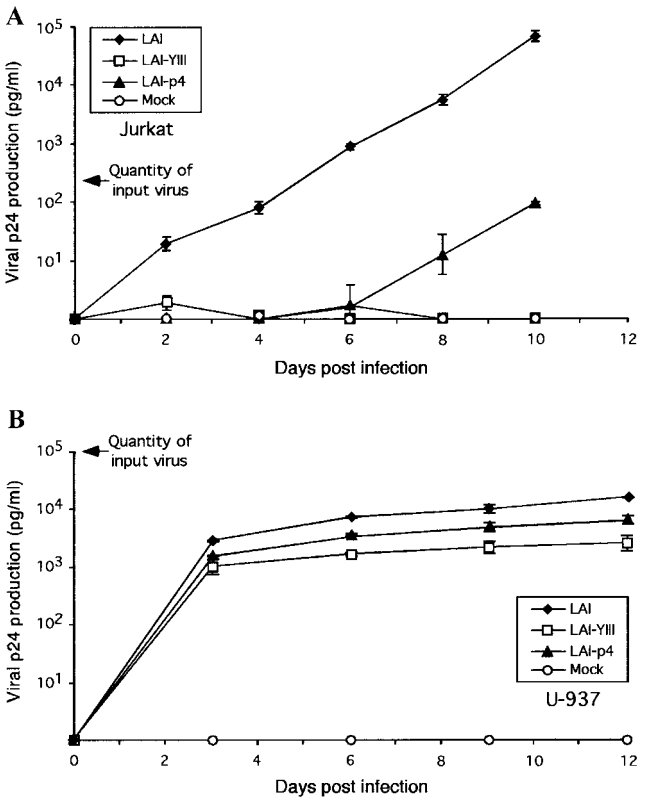


FIG. 4. Replication of HIV-1 molecular clones containing variant Sp site III. Replication of variant viruses in Jurkat T cells and U-937 monocytic cells facilitates a comparison of their replication competence relative to parental LAI. (A) Replication profile resulting from the infection of 3×10^6 Jurkat T cells with 200 pg of input virus. Supernatant samples were taken at 2-day intervals and assayed for p24 production by ELISA. Cells were reseeded into new media at their original plating density at each time point shown. Viruses depicted include parental LAI (LAI), a variant LAI virus containing the YU-2-derived Sp site III (LAI-YIII), and a variant virus containing the p4del Sp site III (LAI-p4del). (B) Viral replication resulting from the infection of 1×10^6 U-937 monocytic cells with 100 ng of input virus. Supernatant samples were taken at 3-day intervals and assayed for p24 production by ELISA. Cells were reseeded into 50% new media at their original plating density at each time point shown. Viruses depicted include parental LAI (LAI), a variant LAI virus containing the YU-2-derived Sp site III (LAI-YIII), and a variant virus containing the p4del Sp site III (LAI-p4del).

virus was found to generate approximately 42% of the p24 produced by the parental LAI molecular clone, whereas the LAI-YIII virus produced 18% of the p24 of the parental virus. Therefore, a comparison of the replication rates of the two viruses in Jurkat T cells and U-937 monocytic cells leads to the conclusion that the absence of a highly functional Sp site III in LAI is of greater detriment in T cells than it is in monocytic cells.

In both cell lines, the replication rate of the mutant viruses correlated with the binding affinity of the Sp site III elements (Fig. 3B), suggesting that differences in viral replication may be caused by differences in Sp factor occupancy at the Sp site III. Because of the prominent roll played by the Sp elements in HIV-1 LTR-directed transcription, cell type-specific differences in viral repli-

cation might be the result of altered basal transcription, Tat-induced transcription, Vpr-induced transcription, or some combination of the three possibilities.

Variant *cis*-acting Sp elements affect basal HIV-1 LTR-mediated gene expression

To examine the impact of the YU-2-derived Sp site III and the p4del natural variant Sp site III on LTR-mediated gene expression, LTR-luciferase constructs were generated for use in transient transfection–reporter gene expression analyses. Utilizing the HIV-1 LAI LTR as a backbone, constructs were generated containing the naturally occurring YU-2-derived Sp site III (LAI-YIII) and the p4del Sp site III (LAI-p4) variants. In addition, the HIV-1 YU-2 LTR was utilized as a backbone to generate constructs containing the LAI-derived (clade B consensus) Sp site III (YU-2-LIII) and the p4del variant Sp site III (YU-2-p4) (Fig. 5A). Each construct was examined for activity in the Jurkat T and U-937 monocytic cell lines. When compared to the activity of the LAI LTR construct, a 58% reduction in LTR activity was observed in Jurkat cells with the introduction of the YIII Sp site III. However, this reduction in activity was found to be less severe in U-937 cells, in which the LAI-YIII construct exhibited a 27% decrease in LTR activity (Fig. 5B; LAI-YIII). These results were consistent with the results obtained in the viral replication studies (Figs. 4A and 4B), in that the detrimental effect of the YIII Sp site III on basal LTR activity is greater in Jurkat cells than in U-937 cells. When the LTR containing the p4del Sp site III was examined in the two cell lines, activity in both lines was reduced by approximately 34% (Fig. 5B; LAI-p4).

The YU-2 LTR was utilized to examine the effect of a high-affinity NF-κB-proximal *cis*-acting Sp element on activity of an LTR which naturally contains a weak Sp element. Unexpectedly, a strong Sp site III appears to be detrimental to YU-2 LTR activity in U-937 cells. The introduction of the LIII Sp site III decreased YU-2 LTR activity in U-937 cells by 61%. In contrast, the high-affinity LIII Sp site increased YU-2 LTR activity fivefold in Jurkat cells (Fig. 5C; YU-2-LIII). In U-937 cells, introduction of the p4del variant Sp site III into the YU-2 LTR generated little change in activity from the parental YU-2 LTR (Fig. 5C; YU-2-p4). This minor difference in activity was consistent with the small difference in binding affinity between the two Sp elements (YIII and p4del). However, the difference was more significant when the same constructs were compared in the Jurkat cell line. Here, the activity of the YU-2 construct was increased twofold in the presence of the LIII Sp site (Fig. 5C; YU-2-p4). Overall, the results generated from the YU-2-based LTR constructs suggest that a high-affinity NF-κB-proximal Sp site III is important for basal LTR function in Jurkat T cells, but detrimental in U-937 monocytic cells. In addition, it suggests that the YU-2 LTR may be regulated differently than the LAI LTR.

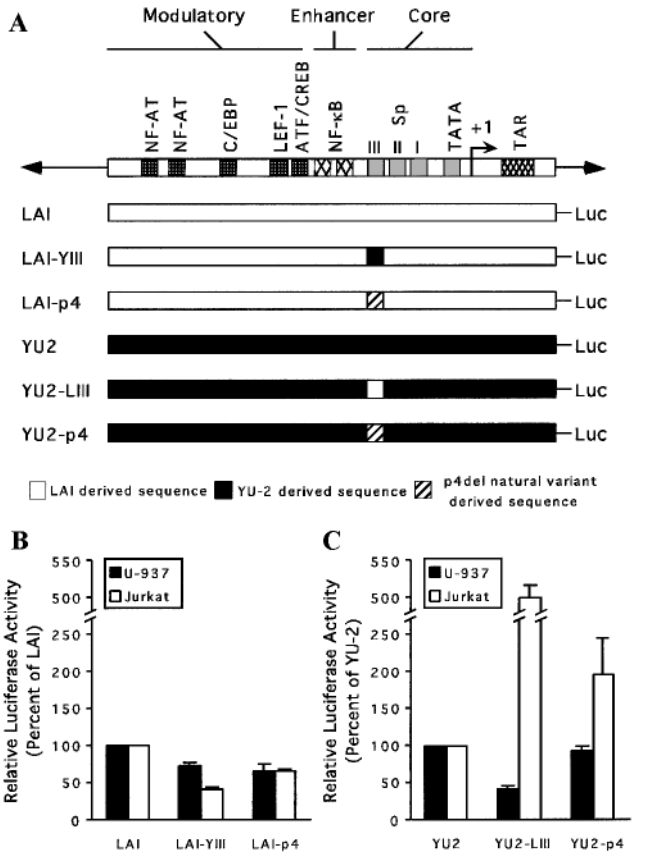


FIG. 5. Transient expression analysis of Sp site III variants in the context of the HIV-1 LAI and YU-2 LTRs. (A) Diagrammatic depiction of the LTR-luciferase constructs examined. Standard reporter gene assays utilizing the dual luciferase reporter system (see Materials and Methods for details) were employed to determine the effect of the YU-2-derived Sp site III (LAI-YIII), and the p4del natural variant Sp site III (LAI-p4) on parental LAI (LAI) LTR function (B). In addition, the effects of the LAI and p4del Sp sites in the context of the YU-2 LTR were also examined (C). Results comparing the activity of constructs in both the Jurkat T cell line and the U-937 monocytic line are shown as a percentage of parental LTR activity. Raw luminescence readings for the LAI construct ranged between 75,824 and 117,725 as compared to background reading, which ranged between 50 and 53 in Jurkat cells. CMV-pRL Renilla values for those same transfections ranged from 65,764 to 112,968 as compared to background readings, which ranged from 1788 to 2002. In U-937 cells, LAI LTR-luc ranged from 7430 to 19592 relative to background readings, which ranged between 43 and 52, whereas TK-pRL ranged from 18,791 to 38,368 as compared to background levels of 1500 to 2149.

Variant *cis*-acting Sp elements affect Vpr-induced HIV-1 LTR-mediated gene expression

Utilizing the same series of LTR-luciferase constructs shown in Fig. 5A, the impact of the NF-κB-proximal Sp element was examined with respect to LTR transactivation by the HIV-1 accessory gene *vpr.* Since previous reports have indicated that Vpr-driven LTR transactivation is mediated through the G/C box array of the HIV-1

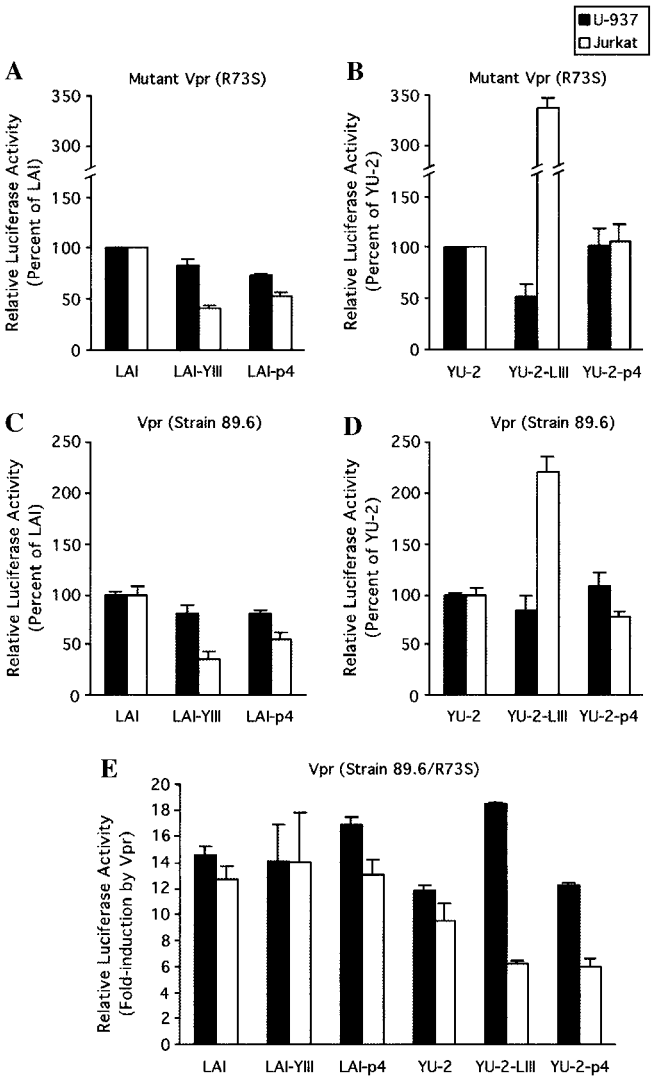


FIG. 6. Transient expression analysis of the Sp site III variants during transactivation by Vpr. Standard reporter gene assays utilizing the dual luciferase reporter system (see Materials and Methods for details) were employed to determine the effect of cotransfected Vpr on the YU-2-derived Sp site III (LAI-YIII), and the p4del natural variant Sp site III (LAI-p4) in the context of parental LAI (LAI) LTR function (A and C). In addition, the effects of Vpr cotransfection on the clade B consensus (YU-2-LIII) and p4del natural variant (YU-2-p4) sites in the context of the YU-2 LTR, which naturally carries the YU-2-derived Sp site III, were examined (B and D). A comparison of activity of the constructs in both the Jurkat T cell line and the U-937 monocytic line in the presence of cotransfected Vpr R73S is shown (A and B). R73S is a mutant Vpr construct, which exhibited no transactivating capacity for the HIV-1 LTR; thus this figure approximates the basal activity shown in Fig. 5. Results are shown as a percentage of parental LTR activity. (C and D) A comparison of the ability of cotransfected Vpr to transactivate each of the variant LTR constructs is shown in both the Jurkat T cell and the U-937 monocytic cell lines. Results are presented as a percentage of Vpr-induced parental LTR activity. (E) The fold-induction by Vpr of each variant Sp site III construct is shown. Fold-induction was calculated by dividing LTR activity in the presence of Vpr 89.6 by LTR activity in the presence of Vpr R73S. Raw luminescence readings for the LAI construct ranged between 107,538 and 130,206 (uninduced) and 1,736,160 and 2,080,572 (induced) as compared to background reading, which ranged between 44 and 564 in Jurkat cells. CMV-pRL Renilla values for those same transfections ranged from 49,098 to 103,710 as compared to background readings, which ranged from 1891 to 2639. In U-937 cells, LAI LTR-luc ranged from 22,266 to 38,187 (uninduced) and 283,941 to 450,663 (induced) relative to background readings, which ranged between 57 and 72, whereas TK-pRL ranged from 56,064 to 77,727 as compared to background levels of 2148 to 2495.

LTR (Wang *et al.,* 1995), it follows that constructs containing variant Sp site III elements might exhibit altered levels of Vpr induction. It was further hypothesized that Vpr might induce transactivation of the LTRs containing the YIII and p4del Sp site III configurations in a cell type-specific manner.

The HIV-1 LAI- and YU-2-based constructs were examined in cotransfection experiments utilizing expression constructs for Vpr (strain 89.6) and a mutant Vpr (R73S) containing an amino acid substitution known to inactivate Vpr-mediated transactivation of the HIV-1 LTR (Sawaya *et al.,* 1999). These studies indicated that R73S Vpr did not significantly alter the relative activities of the LTR-luciferase constructs examined (compare Figs. 6A and 6B to 5B and 5C). In the presence of the transactivating Vpr, LAI LTR activity was elevated 12.8-fold in Jurkat T cells and 14.5-fold in U-937 cells (Fig. 6E; LAI). Relative activities of LAI, LAI-YIII, and LAI-p4 were similar to those observed under nontransactivating conditions (compare Figs. 6C and 6A). A small reduction (19%) in LAI LTR activity was observed with introduction of the YIII Sp site III in U-937 cells, whereas a larger reduction in LAI activity (64%) was observed in Jurkat cells (Fig. 6C; LAI-YIII). Similarly, when the p4del Sp site III was examined, a 19% reduction in LAI LTR activity was observed in U-937 cells, whereas a 45% reduction was observed in Jurkat cells (Fig. 6C; LAI-p4).

The YU-2 LTR-based constructs were also examined in the presence of Vpr. In U-937 cells, YU-2 LTR activity was 24% lower than LAI LTR activity, but the presence of Sp site III variants had little effect on YU-2 activity in the presence of Vpr (Fig. 6D; YU-2, YU-2-LIII, and YU-2-p4). Conversely, the Sp site III variants had a greater affect on YU-2 LTR activity in the Jurkat T cell line. Although YU-2 LTR activity was 56% lower than that exhibited by the LAI LTR, introduction of the Sp site III from the LAI LTR into the YU-2 construct (YU-2-LIII) increased LTR activity by about twofold. Introduction of the p4del Sp site III had little effect on YU-2 LTR activity in the presence of Vpr. In summary, these results suggest that in the presence of Vpr, the activity of LAI and YU-2 LTR is more dependent on a high-affinity *cis*-acting Sp site III in Jurkat T cells than in U-937 monocytic cells. These results are consistent with the observation that the recombinant HIV-1 LAI molecular clones containing the YU-2- and p4-derived natural variants of the Sp site III replicated closer to parental levels in U-937 cells as compared to replication in Jurkat cells.

Results were also expressed as Vpr-mediated fold-induction for each of the constructs examined. For each

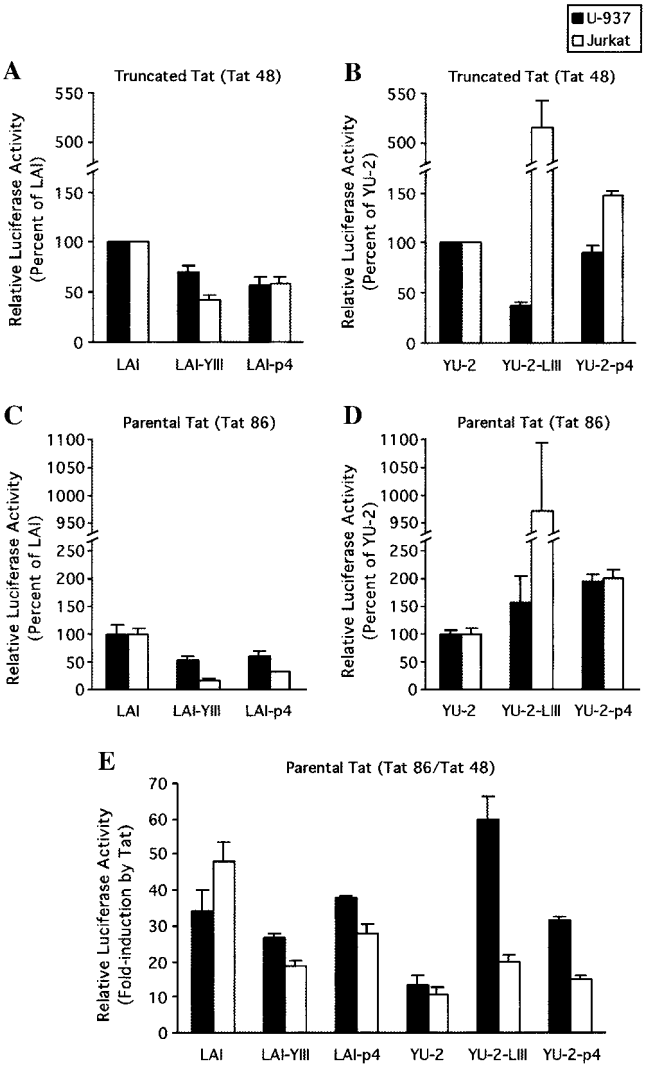


FIG. 7. Transient expression analysis of the Sp site III variants during transactivation by Tat. Standard reporter gene assays utilizing the dual luciferase reporter system (see Materials and Methods for details) were employed to determine the effect of cotransfected Tat on the YU-2-derived Sp site III (LAI-YIII), and the p4del natural variant Sp site III (LAI-p4) in the context of parental LAI LTR (LAI) function (A and C). In addition, the effects of Tat cotransfection on these sites in the context of the YU-2 LTR, which naturally carries the YU-2-derived Sp site III, were examined (B and D). A comparison of the activity of constructs in both the Jurkat T cell line and the U-937 monocytic line in the presence of cotransfected Tat 48 is shown (A and B). Tat 48 is a truncated Tat construct which exhibits no transactivating capacity for the HIV-1 LTR; thus this figure approximates the basal activity shown in Fig. 5. Results are shown as a percentage of LAI LTR activity. A comparison of the ability of cotransfected Tat to transactivate each of the variant LTR constructs is shown in both the Jurkat T cell and the U-937 monocytic cell lines (C and D). Results are presented as a percentage of Tat-induced LAI LTR activity. (E) The fold-induction by Tat of each variant Sp site III construct is shown. Fold-induction was calculated by dividing LTR activity in the presence of Tat 86 by LTR activity in the presence of Tat 48. Raw luminescence readings for the LAI construct ranged between 54,437 and 69,604 (uninduced) and 1,531,024 and 2,127,863 (induced) as compared to background reading, which ranged between 42 and 49 in Jurkat cells. CMV-pRL Renilla values for those same transfections ranged from 49,108 to 98,132 as compared to background readings, which ranged from 2127 to 2250. In U-937 cells LAI LTR-luc ranged from 5302 and 46,073 (uninduced) and 2,241,562 and 3,086,130 (induced) relative to background readings, which ranged between 66 and 80, whereas TK-pRL ranged from 12,608 to 111,694 as compared to background levels of 2811 to 3201.

of the LAI-based constructs (LAI, LAI-YIII, and LAI-p4) fold-induction by Vpr was very similar (14- to 16-fold) in both Jurkat and U-937 cell lines (Fig. 6E; LAI, LAI-YIII, and LAI-p4). This implies that cell type-specific differences in the overall level of Vpr-induced activity (Figs. 6C and 6D) are largely the result of differences in the uninduced activities of the LAI-based constructs. However, in contrast to the LAI constructs, the YU-2-based constructs exhibit different patterns of Vpr-mediated induction. The YU-2 LTR exhibits levels of induction similar to the LAI-based constructs in both cell lines examined (Fig. 6E; compare LAI and YU-2). The induction of the YU-2-LIII construct, however, exhibits a strikingly different level of Vpr-mediated induction in Jurkat and U-937 cells. In Jurkat T cells, YU-2-LIII activity was increased 6.2-fold, whereas in U-937 cells, YU-2-LIII activity was increased 18.5-fold. In a similar manner, the fold-induction of the YU-2-p4 construct was greater in U-937 cells than in Jurkat T cells (Fig. 6C; YU-2, YU-2-LIII, YU-2-p4). Therefore, sequence variants of Sp site III that dramatically alter Sp factor binding in EMS analysis not only affect basal LTR activities in a cell type-dependent manner but, within specific LTR contexts, these elements also may change the degree of Vpr-mediated transactivation.

Variant *cis*-acting Sp elements affect Tat-induced HIV-1 LTR-mediated gene expression

Utilizing the same series of LTR-luciferase constructs shown in Fig. 5A, the effect of the *cis*-acting Sp element on LTR transactivation by the viral transactivator protein Tat was examined. Since previous reports have indicated that Tat-mediated transactivation was partially mediated through the G/C box array of the HIV-1 LTR (Berkhout and Jeang, 1992), it was hypothesized that configurations of the NF-κB-proximal Sp site III exhibiting poor factor recruitment would exhibit weaker responses to Tat-mediated LTR activation.

The HIV-1 LAI- and YU-2-based constructs were examined in cotransfection experiments utilizing expression constructs for a transactivating Tat (Tat 86) and a nontransactivating Tat (Tat 48). Expression of Tat 48 did not significantly alter the relative activities of the LTR-luciferase constructs examined by transient expression (compare Figs. 7A and 7B to 5B and 5C). Expression of Tat 86 induced LAI LTR activity by 34-fold in U-937 cells and by 48-fold in Jurkat cells (Fig. 7E; LAI). In the presence of Tat 86, LAI LTR activity was greatly reduced upon introduction of the YU-2-derived Sp site III. Specifically, a 45% reduction in activity was observed in U-937 cells, whereas an 83% reduction in activity was observed in

Jurkat T cells (Fig. 7C; compare LAI and LAI-YIII). Similarly, the p4del variant Sp site III resulted in a 36% reduction in LAI LTR activity in U-937 cells and a 66% reduction in Jurkat cells (Fig. 7C; compare LAI and LAI-p4). When the YU-2 LTR construct was examined for activity in the presence of Tat, a result similar to that obtained with the LAI-YIII construct was observed. In U-937 cells, YU-2 LTR activity was 69% lower than that exhibited by the LAI LTR. Similarly, in Jurkat cells YU-2 LTR activity was 90% lower than LAI LTR activity. The introduction of a high-affinity *cis*-acting Sp element (Jones *et al.,* 1986) into the YU-2 LTR generated a moderate increase in YU-2 LTR activity in U-937 cells, while producing a much larger increase in Jurkat T cells. In U-937 cells, YU-2 LTR activity increased 61% when the LIII Sp element was introduced. In Jurkat T cells, YU-2 LTR activity increased approximately 10-fold (Fig. 7D; YU-2-LIII). Similar to other constructs containing weakly reactive *cis*-acting Sp site III variants, the p4del variant exhibited a greater reduction in activity in the Jurkat cell line than in the U-937 cell line (Fig. 7D; YU-2-p4). Consistent with the replication characteristics of the recombinant HIV-1 molecular clones containing the YIII and p4 Sp site III variants, these observations indicate an increased dependence on the high-affinity *cis*-acting Sp site III in Jurkat T cells as compared to U-937 monocytic cells.

Results were also expressed as Tat-mediated fold-induction for each of the constructs examined. With but one exception, constructs containing high-affinity Sp elements (LAI and YU-2-LIII) supported greater fold-activation than did their same LTR counterparts containing lower-affinity elements (LAI-YIII, LAI-p4, YU-2, and YU-2-p4), when compared within a given cell type. However, the degree to which this effect was observed was influenced by the cell type. In Jurkat cells, Tat-mediated fold-induction of the LAI LTR was reduced approximately twofold by the presence of low-affinity Sp variants, whereas in U-937 cells, Tat-mediated fold-induction was relatively unaffected by Sp site III variants [Fig. 7E; compare fold-activation in the Jurkat cell line (LAI, 48-fold; LAI-YIII, 18-fold; and LAI-p4, 28-fold) to fold-activation in the U-937 cell line (LAI, 34-fold; LAI-YIII, 27-fold; and LAI-p4, 38-fold)]. Similarly, but reversed with respect to cell type, Tat-mediated fold-induction of YU-2 construct was only moderately affected by Sp site changes in Jurkat cells compared to the much greater effect observed in U-937 cells [Fig. 7E; compare fold-activation in the Jurkat cell line (YU-2, 11-fold; YU-2-LIII, 20-fold; and YU-2-p4, 15-fold) to fold-activation in the U-937 cell line (YU-2, 14-fold; YU-2-LIII, 60-fold; and YU-2-p4, 32-fold)]. This evidence implies that sequence alterations in Sp site III can alter the ability of Tat to amplify LTR activity in addition to affecting basal LTR function. Moreover, the cell type-specific function of Sp site III sequence variants was mediated during both basal and Tat-induced transcription.

DISCUSSION

During the course of an HIV-1 infection, genetic variants rapidly evolve based on extensive viral replication coupled with the high mutational frequency of the reverse-transcriptase enzyme and recombination between diploid genomes. At any given time, this genetic plasticity is being shaped by selective pressures of immunological, physiological, and pharmacologic origin. Together, these forces generate a virus, which rapidly adapts to its environment and has been described as swarms of genetically distinct variants existing simultaneously within single individuals.

Although significant evidence suggests that the viral envelope adapts to particular cell populations (as reviewed by Hoffman and Doms, 1999), less support has been developed for the idea of LTR evolution and its role in pathogenesis and disease progression. In the mouse, retroviral LTR sequences have been demonstrated to influence infection and disease within the CNS (Portis *et al.,* 1990). In the HIV-1 system, Ait-Khaled and colleagues (1995), in a study involving LTR sequences derived from multiple tissues from a single individual, suggested that LTRs may evolve in a tissue-compartmentalized manner. Evidence that LTR sequences derived from brain are functionally distinct from blood-derived LTRs also exists. Corboy and colleagues (1992) demonstrated in a transgenic mouse model that CNS-derived LTRs drove expression of a reporter gene in the CNS, whereas a blood-derived LTR did not. Phylogenetic analysis of a number of LTRs derived from brain tissue of four individuals, however, did not reveal any simple association between specific nucleotides of brain-derived sequences and the CNS clones used in the transgenic experiments, making it difficult to ascribe CNS-specific function to specific *cis*-acting transcriptional control elements within the LTR (Corboy and Garl, 1997). However, because a variety of sequence alterations may result in similar phenotypic effects involving a particular *cis*-acting element (e.g., increased/decreased factor recruitment, or enhanced/reduced affinity for an alternate factor), it is not surprising that simple associations between a particular nucleotide alteration and specific LTRs are difficult to identify based on sequence analysis alone. Rather, one might expect that a constellation of sequence alterations producing similar phenotypes would be selected for during adaptation to new environments. Hence, reports which exclude the possibility of cell type-specific LTR adaptation, based on simple nucleotide comparisons in the absence of any biochemical or functional studies, may be excluding an important element from their analyses.

The impact of the relative affinity of the NF-κB-proxi-

mal Sp element for members of the Sp family of transcription factors on viral replication and gene expression was examined in two cell lines selected as models of the two most prominent cell populations that support viral replication during the natural course of HIV-1 infection. Recombinant HIV-1 molecular clones containing low-affinity Sp elements at site III exhibited significant reductions in the magnitude of viral production coupled with an increased lag before detectable levels of virus were produced in the Jurkat T cell line. The same viral constructs exhibited only minor reductions in the magnitude of viral production during replication in U-937 monocytic cells. These results were supported by studies utilizing LTR-driven reporter genes, which demonstrated a high-affinity Sp site III element was of greater importance to LTR function in Jurkat T cells than in U-937 monocytic cells, and by studies of Vpr- and Tat-mediated LTR activation. In summary, these experimental observations indicate that the cellular environments present in the Jurkat T cell and U-937 monocytic cell vary with respect to their ability to support transcription from HIV-1 LTRs containing alterations which reduce Sp factor recruitment at the NF-κB-proximal Sp element. The differences in the transcriptional environment between these two cell types provide a potential opportunity for HIV-1 to alter its LTR structure through sequence variation and selective pressure to better utilize the transcriptional environment present in each cell type. The Sp site III variants examined exhibited reduced viral replication in both cell types. However, the difference in the magnitude of this effect indicated that alterations to the Sp site III were better tolerated in the U-937 cell line and, since U-937 cells are a model for cells of the monocytic lineage, were possibly better tolerated in monocytic cells. In addition, if the LTR evolves secondary to envelope-mediated tropism, one might expect that the frequency of variant Sp elements found in LTRs derived from M-tropic viruses would exceed the frequency of such changes in T-tropic viruses. This hypothesis may provide an interesting avenue for the investigation of cell type-specific LTR evolution. However, because of the paucity of available confirmed M-tropic LTR sequences, directly assessing this prediction is currently difficult to any degree of statistical certainty.

It is interesting to consider what aspects of the transcriptional environment within the Jurkat and U-937 cell lines may account for the difference in the behavior of the Sp variant viruses between the cell lines. It is intuitive to first consider potential differences in the levels of Sp factors present in each cell type. Although Sp factors were long thought to be basal transcription factors, whose purpose was the steady direction of housekeeping gene expression, recent evidence has revealed an increased complexity within the Sp family of transcription factors. The Sp family of transcription factors now includes Sp1, Sp2, Sp3, and Sp4 (Hagen *et al.,* 1992; Kingsley and Winoto, 1992). Sp1, Sp3, and Sp4 share near-equivalent affinity for binding G/C-rich sequences, whereas Sp2 prefers binding to an alternative G/T-rich sequence (Hagen *et al.,* 1992, 1994; Kingsley and Winoto, 1992). Sp1 and Sp3 are ubiquitously expressed, whereas Sp4 has been shown to be expressed in a brain-restricted fashion (Hagen *et al.,* 1992). With respect to HIV-1, Sp1 and Sp4 have been shown to function as activators of LTR-driven gene expression, whereas both the full-length Sp3 and truncated Sp3 isoforms have been shown to act as repressors (Majello *et al.,* 1994). Hence, competition between Sp1 and Sp3 for a given *cis*-acting Sp element within the HIV-1 LTR could determine whether an activating or repressing activity is realized. Differences in the relative amounts of Sp1 and Sp3 present in different cell types could help determine whether an activating or repressing factor dominates with respect to a specific *cis*-acting element. This potential explanation has been examined and it has been determined that the binding of Sp1 relative to both full-length and truncated Sp3 remains essentially unchanged by EMS analysis between Jurkat T cells and U-937 monocytic cells (Millhouse *et al.,* 1998; McAllister *et al.,* in preparation). However, these results do not exclude the possibility that posttranslational alterations such as phosphorylation or glycosylation (Jackson and Tijan, 1988; Jackson *et al.,* 1990) could impact function subsequent to binding. Although little is known about how these modifications may impact Sp factor recruitment and activity, differences in the levels of such modifications between the cell lines might be associated with the differences in replication of the variant viruses.

A second possibility is that the presence or absence of an Sp factor at the NF-κB-proximal Sp element might exert varying influences on the ability of the LTR to utilize NF-κB within the two cellular environments. This idea is supported by the finding that the induction of LTR activity by various mitogens has been shown to be dependent on protein–protein interactions between NF-κB and Sp1 factors (Perkins *et al.,* 1993). Finally, it is possible that alterations in factors other than those of the Sp family could account for the alteration in activity of the Sp site variant viruses. If LTR activity in one cell type is more dependent on the basal activation provided by the *cis*-acting Sp elements, then the impact on LTR function generated by disrupting Sp factor recruitment at a single site would be significant. Conversely, if LTR activity in a given cell type is less dependent on Sp factors as a result of better support by other transcriptional factors, such as NF-κB or C/EBP, then the impact of a weak *cis*-acting Sp element might be minimized. Studies utilizing mutant viral constructs containing Sp site deletions in peripheral blood leukocytes (PBLs) and T cell lines indicate that replication of virus lacking one or more Sp elements yields a hierarchy of permissiveness (Ross *et al.,* 1991; Parrott *et al.,* 1991). PBLs and MT4 cells exhibited the most permissiveness, while CEM and Jurkat

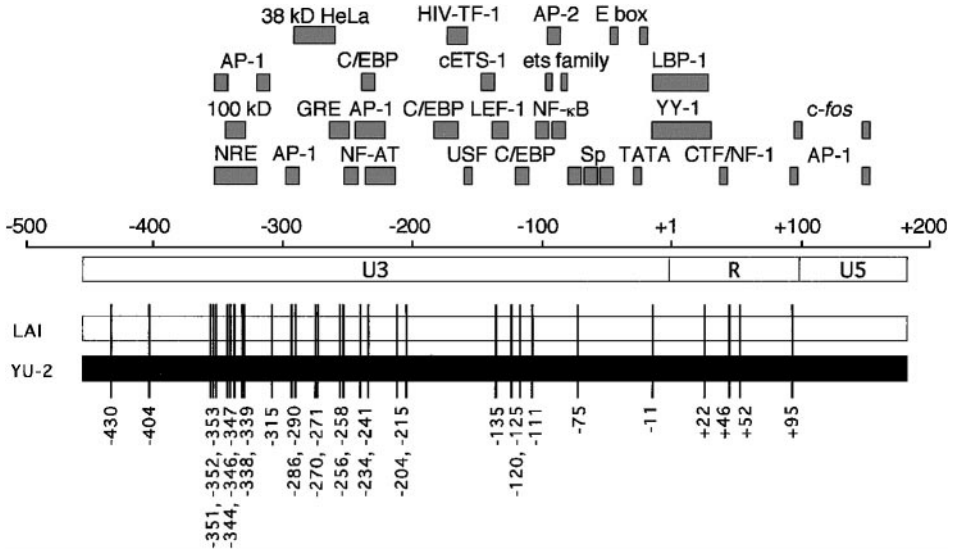


FIG. 8. Sequence comparison of the HIV-1 YU-2 and LAI LTRs. Diagram depicting a sequence comparison of the YU-2 and LAI LTRs. Nucleotide alterations (substitutions and insertions/deletions) are depicted as vertical lines labeled by their location relative to the start site of transcription. These alterations are shown in relation to the location of numerous *cis*-acting elements found in the HIV-1 LTR (*cis*-acting elements adapted from Krebs *et al.,* 1998) for rapid comparison.

cells exhibited the least. These studies suggested that the hierarchy seen likely reflects the relative abundance of transcription factors present in each cell type relative to the available *cis*-acting elements of a given LTR. These results imply that low levels of NF-κB in unstimulated Jurkat T cells might influence the impact of the altered *cis*-acting Sp element. In addition, utilization of the C/EBP family of transcription factors by HIV-1 LTRs in monocytic cells may reduce the dependence on Sp and NF-κB factors observed in T cells. Despite the importance of C/EBP factors with respect to HIV-1 LTR function in monocytes (Henderson and Calame, 1997), such an effect is unlikely in T cell lines since EMS analysis has demonstrated that Jurkat T cells exhibit less C/EBP binding activity than do U-937 monocytic cells (data not shown).

Another interesting observation in the studies reported herein was the resultant basal activity of the YU-2 LTR after the normally weak NF-κB-proximal Sp element was replaced by a strong (clade B consensus) Sp element. The reporter gene activity of the YU-2 LTR increased from 60 to 300% of the activity of the LAI LTR with the introduction of the clade B consensus Sp site III in Jurkat T cells, which represents a fivefold elevation in basal activity. Conversely, in the U-937 monocytic cells the activity of the YU-2 LTR decreased from 80 to 25% of the activity of the LAI LTR. These observations indicated that for basal LTR-driven gene expression a strong Sp site III element was detrimental in the U-937 cell line, while greatly advantageous in Jurkat T cells. Interestingly, this result was not paralleled in the LAI LTR. Although introduction of the YU-2-derived Sp site III into the LAI LTR generated a 60% decrease in basal activity in Jurkat T cells, the same alteration did not generate an increase in LTR activity when expressed in U-937 cells. These studies indicate inherent differences in the LTR backbones, which influence the effects mediated through the variant Sp elements. Alignment of the LAI and YU-2 LTRs revealed 31 nucleotide alterations, counting both substitutions and insertions/deletions (Fig. 8). With the exception of the aforementioned G:A change in the NF-κB-proximal Sp element (position −75 relative transcriptional initiation), no changes were noted in the core or enhancer regions. Within the upstream modulatory region a number of groupings of alterations were observed. First, a group of four alterations were found between −110 and −135, a region occupied by *cis*-acting elements for C/EBP and LEF-1. A second large grouping of alterations was observed between −200 and −350, a region containing numerous *cis*-acting elements, including NF-AT, C/EBP, AP-1, and the NRE. There is a strong concentration of alterations grouped within the negative regulatory element (NRE, −320 to −356), a region that normally exerts a repressive effect on LTR-mediated transcription (Lu *et al.,* 1990). Specifically, eight nucleotides were misaligned as a result of insertions/deletions totaling five nucleotides and base-pair substitutions at three positions. These changes may reduce the effect of the NRE on YU-2 LTR function to partially compensate for loss of a strong Sp site III. Such a hypothesis could then be used to explain the strong elevation in basal activity generated by the introduction of the clade B consensus Sp site III to the YU-2 LTR. Future studies will further examine this hypothesis.

With respect to Tat transactivation, Sp site function was also examined in the context of the YU-2 and LAI

LTRs. Consistent with previous studies (Harrich *et al.,* 1989; Kamine *et al.,* 1991) high-affinity Sp site III elements in both the LAI and YU-2 LTRs yielded greater fold-activation than did lower-affinity elements in a given cell type. Tat-mediated activation of these LTRs was modulated by both the relative affinity of the Sp site III element and cell type in which they were expressed (Fig. 7E). Interestingly, differences in YU-2 LTR activation among the Sp site III variants was more apparent in U-937 cells, whereas differences in LAI LTR activation among the Sp site III variants was more apparent in Jurkat cells. As with basal activity, these findings imply inherent functional differences in the YU-2 and LAI LTRs. These observations imply that Tat-mediated induction is dependent on the affinity of the Sp site III element, and that cell type-specific differences in LTR activity observed in the presence of Tat were driven by Tat transactivation in addition to basal transcription.

In contrast to Tat, Vpr-mediated transactivation of the LAI LTR was unaffected by the relative affinity of the Sp site III element studied (Fig. 6E). However, Vpr-mediated induction of the YU-2 LTR exhibited cell-type specificity, depending on the relative affinity of the Sp site III element present (Fig. 6E). Hence, in the context of the LAI LTR, differences in the activity of Sp site variants in the presence of Vpr are primarily the result of cell type-specific differences in basal activity. Alternatively, in the context of the YU-2 LTR, differences in the activity of Sp site variants are more likely generated by cell type-specific effects in Vpr-mediated induction as well as basal transcription. Although Vpr has been shown to mediate its transcriptional effects through the Sp array (Wang *et al.,* 1995), the Sp site-dependent differences in Vpr-mediated induction observed in the context of the YU-2, but not the LAI LTR, may imply that elements outside of the Sp array also influence Vpr-mediated activation in certain HIV-1 LTRs. Modulation of Vpr-mediated LTR activity could be the result of a direct interaction between Vpr and a given *cis*-acting element, or a protein/protein interaction between Vpr and a non-Sp transcription factor. One potential candidate for such a Vpr-modulating interaction is the C/EBP family of transcription factors. Differences in *cis*-acting C/EBP elements between the two LTRs (Fig. 8), coupled with the cell type-restricted pattern of C/EBP expression, make this an intriguing possibility.

In summary, differences in the utilization of the Sp site III element between the Jurkat T cell line and the U-937 monocytic cell line illustrate a potential avenue for LTR sequence variation and adaptation to influence cell type-specific viral replication. Inherent differences in gene regulation between the T-tropic HIV-1 LAI-derived LTR and the M-tropic HIV-1 YU-2-derived LTR further support this hypothesis. However, additional studies are required to fully elucidate the potential impact of the HIV-1 LTR on cell type-specific viral replication and gene regulation.

MATERIALS AND METHODS

Cell culture and nuclear extract preparation

The human monocytic line U-937 and the human T cell line Jurkat were grown in RPMI 1640 media (Sundstrom and Nilsson, 1976; Nagasawa *et al.,* 1981). HeLa cells were grown in Delbecco's media (Jones *et al.,* 1971). All media were supplemented with 10% heat-inactivated fetal bovine serum, antibiotics (penicillin, streptomycin, and kanamycin at 0.04 mg/ml each), L-glutamine (0.3 mg/ml), and sodium bicarbonate (0.05%). All cell lines were maintained at 37°C in 90% relative humidity and 5% CO_2. Nuclear extracts were prepared (Dignam *et al.,* 1983) and quantitated for protein concentration as described (Bradford, 1976). Briefly, exponentially growing cells were collected by centrifugation, swelled hypotonically, and lysed by Dounce homogenization. Nuclei were pelleted and lysed to extract nuclear proteins, which were dialyzed in buffer [HEPES, pH 7.9 (20 mM), glycerol (20% v/v), KCl (100 mM), EDTA (0.2 mM), PMSF (0.2 mM), and DTT (0.5 mM)] for 30 min at 4°C.

Oligonucleotide probe synthesis and labeling

Complementary single-stranded oligonucleotides were synthesized (Macromolecular Core Facility, Penn State College of Medicine, Hershey, PA) and annealed by heating to 95°C followed by gradual cooling to room temperature. The resulting double-stranded oligonucleotides were gel-purified and radiolabeled using T4 polynucleotide kinase (Gibco BRL, Gaithersburg, MD) and [γ-^{32}P]ATP. The sequences of the probes used in these studies were as follows: LIII (LAI Sp site III), CCAGGGAGGCGTGGCCTGGG; YIII (YU-2 Sp site III), CCAGGGAAGCGTGGCCTGGG; p4del (p4 variant Sp site III) TCCAGGGAGCGTGGCCTGGG; con BI (clade B consensus Sp site I) GGACTGGGGAGTGGCGAGCC.

EMS analyses

EMS analyses were performed as previously described (Garner and Revzin, 1981). Radiolabeled probe was incubated with nuclear extract (6 μg) and poly (dI-dC, 1 μg) for 20 min at 30°C. DNA–protein complexes were resolved by electrophoresis for 2 h at 30 mA and 4°C in a 4% nondenaturing polyacrylamide gel, which was subsequently dried at 80°C for 45 min, and subjected to autoradiography. Competition EMS analyses were conducted by adding the indicated molar excess of unlabeled homologous or heterologous oligonucleotide probe.

Plasmid construction and site-directed mutagenesis

The parental LAI LTR, obtained from Dr. Maureen Goodenow (University of Florida, Gainesville, FL), was cloned into the pGL3-Basic Vector (Promega, Madison, WI) to construct LAI-Luc. The parental YU-2 LTR was

obtained by Pfu-mediated PCR amplification of the YU-2 molecular clone (Li *et al.,* 1991), obtained from Dr. Dana Gabuzda (Harvard University, Dana-Farber Cancer Institute, Boston, MA). Specifically, primers TGCACTGCAGACTGGAAGGGCT and CCCTCTAGACTGCTAGAGAT were used to amplify and subsequently clone the LTR into the *Xba*I and *Pst*I sites of a modified multiple cloning region within the pGL3-Basic Vector, generating YU-2-Luc. Both the parental YU-2-Luc and the LAI-Luc constructs were used as templates for site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA) to construct LAI-YIII-Luc, LAI-p4-Luc, YU-2-LIII-Luc, and YU-2-p4-Luc. LAI-YIII-Luc contains a single G to A nucleotide substitution in the NF-κB-proximal Sp site, giving this site the configuration published for the YU-2 LTR (GAAGCGTGGC) (Li *et al.,* 1991) in the context of the LAI LTR. LAI-p4-Luc has a single nucleotide deletion at position four of the published sequence for the LAI-derived Sp site III, giving it the p4del natural variant Sp site III configuration (GGAGCGTGGC) in the context of the LAI LTR. YU-2-LIII-Luc contains the clade B consensus Sp site III (the LAI Sp site III: GAGGCGTGGC) in the context of the YU-2 LTR, whereas YU-2-p4-Luc places the p4del variant in the context of the YU-2 LTR. The sequence of each LTR construct was verified prior to experimentation. In addition, during the generation of each of the mutant constructs described, two or more sequence-confirmed clones were generated and tested for comparable activity. After establishing that the clones behaved equivalently, a single clone was selected for detailed experimentation.

Transient transfections and luciferase assays

Cell lines (U-937 or Jurkat) were seeded from exponentially growing, low-passage cultures at 5×10^5 cells/ml growth medium and transfected with Fugene 6 (Boehringer-Mannheim, Indianapolis, IN) in 35-mm plates (Falcon, Lincoln Park, NJ). Premixed Fugene (94 μl serum-free RPMI with 6 μl Fugene) was added dropwise to either 1.5 or 0.5 μg of the experimental luciferase vector (Jurkat and U-937, respectively) and either 125 ng pRL-CMV or 50 ng pRL-TK Renilla internal control vector (Jurkat and U-937, respectively). After a 15-min incubation, the Fugene/DNA mixture was added directly to the cells. Cultures were incubated at 37°C for 24 h. Cell extracts were harvested and assayed for luminescence using the Femtomaster FB 12 luminometer (Zylux, Pforzheim, Germany) as described for the Dual Luciferase Assay (Promega, Madison, WI). When cotransfection with either Vpr or Tat was performed, the additional expression constructs were included in the Fugene/DNA mixture. Vpr-pcDNA3 (an active construct derived from the HIV-1 89.6 molecular clone) was controlled for by the use of Vpr-R73S-pcDNA3 (mutated nontransactivating construct). In both cases, the Vpr expression constructs were added at 10% the level of the experimental LTR-luciferase vector. The Vpr expression vectors were provided by Dr. Bassel Sawaya (Temple University, Philadelphia, PA). Tat86-pcDNA3 (active construct) transactivation was controlled for by the use of Tat48-pcDNA3 (truncated nontransactivating construct). In both cases, the Tat expression constructs were added at 20% the level of the experimental LTR-luciferase vector. The Tat expression vectors used were provided by Dr. Kamel Khalili (Temple University). Firefly luminescence was normalized to the Renilla luminescence to control for variability in transfection efficiency. The results have been presented with the parental LTR values (LAI-Luc) set to 1.0 for each experiment and the relative activity of mutagenized LTRs was normalized to this value. Each experimental point was performed in duplicate within an experiment. Error bars indicate the standard deviation of data obtained from two independent experiments.

Mutant molecular clone construction

To generate molecular clone constructs containing mutations within their LTRs, a single LTR construct was subcloned from the parental HIV-1 molecular clone. This subclone was then used as a template for site-directed mutagenesis using the QuickChange mutagenesis kit (Stratagene, LaJolla, CA). The single LTR construct was generated by digesting the LAI molecular clone with *Bsp*EI, an enzyme which recognizes a unique site within the LAI LTR. The resulting fragments (a 9-kb HIV-1 genomic fragment and a 3-kb plasmid backbone fragment) were purified. The smaller fragment containing the bacterial plasmid along with two partial LTRs was ligated to yield a single LTR plasmid construct. This construct was then mutated to introduce the YU-2-derived Sp site III (LAI-YIII) and the p4del Sp site III (LAI-p4). Two separate mutagenesis reactions yielded two independently derived clones of each desired mutant. The sequence of all LTR constructs was verified prior to experimentation. The *Bsp*EI site is 5′ relative to the G/C box array and, therefore, reconstruction of full-length molecular clones from the 9-kb genomic fragment and the 3-kb mutated single LTR constructs resulted in molecular clones containing alterations in their 3′ LTRs (a necessity for production of infectious virus containing the desired mutations within the context of the 5′ LTR). Two sequence-confirmed clones of each mutant virus were reconstructed by ligation. Multiple clones of each of the resulting full-length constructs were identified by restriction endonuclease analysis and subsequently tested for the presence of the desired mutation by back-isolating single LTR constructs from the mutant molecular clones themselves and sequencing the LTR. Only mutant molecular clones, which could be sequence-confirmed in this manner, were used to generate primary viral stocks.

Viral replication analysis

Primary viral stocks were generated by transfection of the molecular clone constructs into HeLa cells. Transfection was performed 24 h after plating 1×10^6 target cells into flasks (25 cm^2; Falcon). Lipofectamine (Promega) was mixed with molecular clone DNA (15 μl lipofectamine, 7.5 μg DNA in 300 μl of Optimem) and incubated for 30 min before being added to cells in a final volume of 1.5 ml. After 5 h, the transfection media was washed off and replaced with Delbecco's media. Primary viral stocks were harvested 48 h posttransfection. To ensure that no cells were carried over, the viral stocks were subjected to centrifugation and passed through a 0.45-μm filter (Nalgene). Stocks were aliquoted and frozen at −70°C. The viral titer of a given stock was determined by p24 assay (NEN, Boston, MA) performed according to manufacturer's specifications. Amplification of viral titer was performed by inoculating peripheral blood leukocytes with primary viral stocks and incubating at 37°C for 18 to 21 days in the presence of PHA (5 μg/ml; Sigma, St. Louis, MO) and IL-2 (0.5 ng/ml; R&D Systems, Minneapolis, MN). Amplified viral stocks were harvested and quantitated as described for primary viral stocks. Viral infections were initiated with equal amounts of stock virus, as quantitated by p24 assay. Jurkat and U-937 cells were inoculated with 200 pg and 100 ng virus, respectively, and after a 4-h absorption period the cells were washed with PBS to remove unadsorbed input virus. Supernatant samples were taken every 2 or 3 days (Jurkat or U-937, respectively) and assayed for p24 production. At each time interval, cells were counted by standard trypan blue dye exclusion and plated at their original densities (1.5×10^6 cells/ml, Jurkat; 5×10^5 cells/ml, U-937). The entire volume of replication media for Jurkat cells and half the volume of replication media for the U-937 cells were replaced during the reseeding process.

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